REMARKS

Claims 1-20 and 27-30 are pending in the present application. In light of the following remarks, applicants respectfully request reconsideration of this application, and allowance of the claims to issue.

Applicants appreciate the opportunity to telephonically interview this case on January 21, 2004 with Examiner Landsman. During this interview the rejection of claims 1-20 and 27-30 under 35 U.S.C. § 103(a) was discussed. The following remarks more specifically address the issues discussed in the interview. In light of these remarks, applicants respectfully request reconsideration of this application and allowance of the pending claims to issue

Rejections Under 35 U.S.C. § 103(a)

A. The Office Action states that claims 1-20 and 27-30 remain rejected under 35 U.S.C. § 103(a) for reasons of record. Specifically, claims 1-20 and 27-30 remain rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over White et al. (*Nature* 396: 679-682, 1998) in view of Miyawaki et al. .(*Nature* 388: 882-887, 1997) and further in view of Hebert et al.(*Biochem. Cell Biol.* 76: 1-11, 1998). The Examiner has considered Applicant's arguments, but according to the Examiner, these arguments are not deemed persuasive.

As pointed out during the interview by the inventor, Dr. Kendall Blumer, until recently, the prevailing assumption was that G protein coupled receptors (GPCRs) function *in vivo* as monomeric proteins. However, studies suggesting the existence of dimeric or oligomeric GPCRs were described in the literature (reviewed by Bouvier "Oligomerization of G-Protein Coupled Transmitter Receptors" *Nat Rev Neurosci* 2:274-8 (2001) (previously submitted with Amendment filed on November 7, 2002)). These studies showed that GPCRs could form dimers or oligomers under conditions where receptors were inactive, for example, denaturing SDS polyacrylamide gel electrophoresis or co-immunoprecipitation after receptors were released in inactive form from membranes via detergent extraction. However, these results were not convincing. As

stated in Bouvier, "[a]lthough fairly convincing, co-immunoprecipitation and western-blot analyses require receptor solubilization, raising the possibility that the observed dimers could be solubilization artefacts. This is a putatively important caveat when considering proteins such as GPCRs that are composed of seven hydrophobic transmembrane domains. Incomplete solubilization could easily lead to aggregation that could be mistakenly interpreted as dimerization." (Bouvier, page 267, col. 1, last full paragraph). These concerns were also raised in a review by Angers et al. (*Annu. Rev. Pharmacol. Toxicol.* 2002. 42:409-435) (previously submitted with response filed on July 14, 2003). Specifically, page 410 of the review states that, "coimmunoprecipitation of membrane receptors requires their solubilization using detergents, and it may be problematic when considering highly hydrophobic proteins such as GPCRs that could form artifactual aggregates upon incomplete solubilization." Therefore, studies prior to the present invention only revealed that a biochemical interaction can occur *in vitro*, which may not be biologically relevant. In other words, these interactions, did not show that GPCRs were interacting in the cell membrane of a living cell, much less that GPCRs oligomerize as they carry out a biological function. For this reason, Angers et al. conclude on pg. 411 of their review that, "the general acceptance of GPCR dimerization still awaited the direct demonstration that these complexes existed in living cells." Thus, Angers et al. states explicitly that one skilled in the art would not conclude that GPCR oligomerization would be expected prior to the direct demonstration, i.e., the first real time, *in vivo* evidence of G protein-coupled receptor oligomerization in intact cells. In fact, it was Applicant's invention that provided this direct demonstration, i.e., the first real time, *in vivo* evidence that G protein-coupled receptors oligomerize in living cells as they carry out their biological functions. It is clear that the ability to measure receptor interactions was not possible until the present invention definitively showed that G protein-coupled receptors interact *in vivo*, in intact cells. In fact, Applicant's work has been cited as the first use of FRET (see Bouvier, specifically page 276, second col. first full paragraph) to demonstrate that a GPCR oligomerizes in a living cell when bound by its agonist and transmitting a signal

As mentioned above, the prior art (for example, the White et al. reference) showed GPCRs could form dimers or oligomers under conditions where receptors were

inactive. During the interview, Applicant specifically addressed the studies set forth in the White et al. (*Nature* 396: 679-682, 1998) reference cited by the Examiner. Applicant reiterated that this reference does not provide evidence showing that GPCRs are oligomeric *in vivo* and the following sets forth the limitations in the White et al. studies.

Technically, White et al. provided biochemical evidence (yeast two-hybrid experiments and co-immunoprecipitation experiments) suggesting an interaction between GABA-B(R1) and (R2). However, there are limitations regarding the data showing an interaction between the R1 and R2 subunits of the $GABA_B$ receptor. First, White et al. did not demonstrate that $GABA_B$ receptors were functional or active under the conditions of detergent extraction during immnoprecipitation experiments used to detect interaction between the R1 and R2 subunits (White et al., Figure 2). Second, White et al. did not determine whether the interaction between R1 and R2 occurred inside the cell or on the plasma membrane where the receptor functions. Such information was needed to determine whether $GABA_B$ receptors function *in vivo* (i.e. transmit a GABA signal from the plasma membrane) as a heterodimer composed of R1 and R2 subunits. Third, the authors did not determine whether interaction of the normal, full-length R1 and R2 proteins (determined by co-immunoprecipitation) required their coiled-coil domains. The only evidence implicating an interaction involving these coiled-coil domains came from experiments showing that small pieces of the R1 and R2 proteins could interact when expressed heterologously in the nucleus of yeast cells (White et al., Figure 2a). This result was not physiologically relevant because, in these experiments, only small pieces (coiled-coil domains) of the R1 and R2 subunits were shown to interact. Furthermore, this interaction was irrelevant because it was detected in the nucleus rather than the plasma membrane where $GABA_B$ receptors normally reside and function.

In addition to the limitations regarding the interaction between R1 and R2, there are also limitations regarding the influence of the R2 subunit on the R1 subunit. White et al. clearly showed that R1 expression at the cell surface required co-expression with R2 (White et al., Figure 3). In contrast, R2 could be expressed on the cell surface without R1. These results showed that unknown aspects of R1 biogenesis (such as synthesis, movement from its site of synthesis inside the cell to the plasma membrane where it functions, or stabilization on the plasma membrane) depended directly or indirectly on

co-expression with R2. However, this information did not determine whether a complex of R1 and R2 transmits the GABA signal at the plasma membrane. Therefore, these results did not show that R1 and R2 interact at the plasma membrane to carry out a biological function in a living cell.

Furthermore, as pointed out by Applicant during the interview, there are also limitations in the $GABA_B$ receptor function assays set forth by White et al. White et al. showed that a functional $GABA_B$ receptor was produced following co-expression of R1 and R2 (White et al., Figure 4c). Applicant agrees that Figure 4c showed that GABA produced a biological response (elicitation of an inward current) only when R1 and R2 were expressed together in the same cell. However, Applicant noted to the Examiner that the readout of receptor function in Figure 4c (inward current) did not in any way provide a direct measure of whether R1 and R2 were associated when the biological response was triggered by GABA (i.e. when the receptor was functioning). The same limitation held true for the other panels in Figure 4. In Figure 4a, the readout of receptor function was simply GABA binding. In Figure 4b, the readout of receptor function was G protein activation (GABA-stimulated binding of labeled GTPγS to G proteins). In Figure 4d, the readout again was inward current elicited by GABA. Thus, Figure 4 showed that R1 and R2 must be co-expressed in order to obtain a functional $GABA_B$ receptor. Nevertheless, White et al. did not demonstrate that R1 and R2 form a complex on the plasma membrane while the receptor transmits a signal. Accordingly, when discussing these results at the bottom of pg. 681 the authors qualified their conclusions by stating that "[c]o-expression of $GABA_BR1$ and $GABA_BR2$ *appears* (emphasis added) to be a prerequisite for maturation and transport of $GABA_BR1$ to the plasma membrane and results in high-affinity GABA binding and G-protein activation."

Therefore, White et al., provides only indirect evidence of receptor oligomerization based on *in vitro* results and not definitive evidence that *in vivo* receptor interactions (i.e. oligomerization) occur on the cell membrane, in intact cells, in such a way, that these interactions can be observed, in real time, and accurately measured, while carrying out a biological function.

With respect to Miyawaki et al., for the reasons set forth in the Response filed on July 14, 2003, Applicant reiterates that although Miyawaki et. al.(*Nature* 388: 882-887,

1997) discloses the use of fluorescence resonance energy transfer (FRET) to detect an intramolecular interaction, this reference is not relevant to the detection of real-time, *in vivo* intermolecular interactions, much less an intermolecular interaction between transmembrane proteins, such as GPCRs.

Regarding Hebert et al.(*Biochem. Cell Biol.* 76: 1-11, 1998), although this reference suggests the use of FRET, this reference merely provides a suggestion and it was not until applicants actually obtained meaningful expression resulting in accurate, measurable interactions that FRET could be effectively utilized to study the interaction between GPCRs. Prior to Applicant's invention, it was unknown whether FRET could occur as a result of specific and stable interactions between proteins in the membrane. The applicant has provided extensive evidence to this effect. Such evidence provides an important distinction relative to Hebert et al. which merely suggested the potential utility of FRET to detect GPCR oligomerization. Thus, Hebert et al.'s mere suggestion to use FRET, in the absence of data, does not provide to one of skill in the art a reasonable expectation of success for the real time, *in vivo* detection of GPCR oligomers in living cells via FRET, while they carry out their biological function.

Therefore, prior to Applicant's invention and in light of the limitations of White et al., there was no reasonable likelihood of success that FRET could be utilized to show that GPCRs function as oligomers as they perform a biological function. Therefore, it would not have been obvious for one of skill in the art to combine the inconclusive teachings of White et al., with Hebert et al.'s suggestion and with the teachings of Miyawaki et al., directed to intramolecular interactions to arrive at the present invention. Thus, Applicant believes this rejection has been overcome and respectfully requests its withdrawal.

B. The Office Action states that claims 5 and 10 remain rejected under 35 U.S.C. 103(a) for reasons of record. Claims 5 and 10 are allegedly unpatentable over White et al. in view of Miyawaki et al. (Nature 388:882-887, 1997), further in view of Gama et al. (J. Biol. Chem. 273:29712-29718, 1998) and further in view of Hebert et al.

The teachings of White et al., Hebert et al., and Miyawaka et al. and how it would not have been obvious to combine any of these references to arrive at the present invention are discussed above. Applicant reiterates that although the Office Action contends that there is a high likelihood of success to measure FRET in truncated receptors, the GFP fusion proteins of Gama et al. do not provide any indication as to how fluorescent fusion proteins comprising G protein-coupled receptors can be utilized in a FRET assay. Furthermore, Gama et al. provides no indication that the interaction between G protein-coupled receptors can be measured. Therefore, since there is no suggestion or motivation provided by Gama et al. to study the interactions between G protein-coupled receptors, Gama et al. could not be combined with the inconclusive teachings of White et al, the suggestion of Hebert et al. and the teachings of Miyawaki et al., directed to intramolecular interations, to arrive at the present invention. Thus, applicants believe this rejection has been overcome and respectfully request its withdrawal.

Applicant reiterates that as a result of Applicant's invention, GPCR oligomerization can now be observed, in real time, and measured accurately. Prior to this work, it was not possible to directly measure the oligomerization of G protein-coupled receptors *in vivo*, nor was it possible to measure the effects of compounds on the oligomerization of G protein-coupled receptors or their biological functions *in vivo*. Therefore, the present invention has provided a better understanding of how G protein-coupled receptors interact *in vivo* as well as a better understanding of how compounds that affect these receptors exert their effects *in vivo*, thus providing a significant advance in the field of receptor biology.

In further support of Applicant's breakthrough and the nonobviousness of the present invention, subsequent to the filing date of this application, in addition to the recognition of Applicant's seminal use of FRET with GPCRs in intact living cells (Bouvier, 2001) mentioned above, the FRET approach developed by Applicant has been employed by Applicant and other investigators to show that many GPCRs function as oligomers *in vivo*.

For example, Floyd et al. ("C5a Receptor Oligomerization" *J. Biol. Chem.* 278(37) 35354-35361 (2003)) (Exhibit A, attached hereto) utilized FRET to show that

human C5a receptor forms oligomers *in vivo*. Dinger et al. ("Homodimerization of Neuropeptide Y Receptors Investigated by Fluorescence Resonance Energy Transfer in Living Cells" *J. Biol. Chem.* 278(12):10562-10571 (2003)) (Exhibit B, attached hereto) utilized the teachings of the present invention to show that Neuropeptide Y Receptors form homodimers and remain functional in the cell membrane of a living cell. Hernanz-Falcon et al. ("Identification of amino acid residues critical for chemokine receptor dimerization" *Nature Immunology* Online Publication January: 1-8 (2004)) (Exhibit C, attached hereto) utilized FRET to study the *in vivo* dimerization and function of the CCR5 receptor. Stanasila et al. ("Oligomerization of the α_{1a}- and α_{1b}- Adrenergic Receptor Subtypes" *J. Biol. Chem.* 278(41): 40239-40251 (2003)) (Exhibit D, attached hereto) utilized FRET to study the oligomerization of α_{1a}- and α_{1b}- adrenergic receptor subtypes. Wurch et al. ("Agonist-independent and –dependent oligomerization of dopamine D2 receptors by fusion to fluorescent proteins" *FEBS Letters* 507:109-113 (2001)) (Exhibit E, attached hereto) utilized FRET to show that D2 receptors oligomerize and function in intact living cells.

Therefore, based on the evidence described above, Applicant's invention provided a breakthrough in receptor biology that would not have been obvious to one of skill in the art the time the application was filed. It is clear that those skilled in the art have specifically recognized that without Applicant' s invention, it was not possible to directly measure the oligomerization of G protein-coupled receptors *in vivo*, nor was it possible to measure the effects of compounds on the oligomerization of G protein-coupled receptors or their biological functions *in vivo*.

Comments on Enablement

Applicant notes that a rejection under 35 U.S.C. § 112, first paragraph has not been made by the Examiner. However, during the interview, Examiner Landsmen requested that Applicant comment on the enablement of the claims.

The Office Action stated that if the requirements of Overton and Blumer need to be met for FRET to be detected, then the claims would allegedly need to recite exactly where on the GPCRs, the CFP and YFP would need to be attached in order to detect FRET.

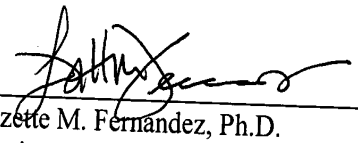
Applicants respectfully disagree and point out to the Examiner that as stated above, subsequent to filing of the present application, the FRET approach developed by Applicant has been employed by Applicant and other investigators to show that many GPCRs function as oligomers *in vivo*. These include, Floyd et al. ("C5a Receptor Oligomerization" *J. Biol. Chem.* 278(37) 35354-35361 (2003)) who utilized FRET to show that human C5a receptor forms oligomers *in vivo*; Dinger et al. ("Homodimerization of Neuropeptide Y Receptors Investigated by Fluorescence Resonance Energy Transfer in Living Cells" *J. Biol. Chem.* 278(12):10562-10571 (2003)) who utilized the teachings of the present invention to show that Neuropeptide Y receptors form homodimers and remain functional in the cell membrane of a living cell; Hernanz-Falcon et al. ("Identification of amino acid residues critical for chemokine receptor dimerization" *Nature Immunology* Online Publication January: 1-8 (2004)) who utilized FRET to study the *in vivo* dimerization and function of the CCR5 receptor; Stanasila et al. ("Oligomerization of the α_{1a}- and α_{1b}- Adrenergic Receptor Subtypes" *J. Biol. Chem.* 278(41): 40239-40251 (2003)) who utilized FRET to study the oligomerization of α_{1a}- and α_{1b}- adrenergic receptor subtypes; and Wurch et al. ("Agonist-independent and –dependent oligomerization of dopamine D2 receptors by fusion to fluorescent proteins" *FEBS Letters* 507:109-113 (2001)) who utilized FRET to show that D2 receptors oligomerize and function in intact living cells.

Therefore, it is clear that the methodology pioneered by Applicant, as set forth in the present application, provided sufficient guidance for those of skill in the art to determine that other GPCRs function as oligomers *in vivo*, without specific requirements for the type of GPCR that can be utilized or where the fluorescent donor and acceptor should be attached on the GPCR. Based on the teachings of the present application, it is now routine to make GPCR fusion proteins comprising any GPCR and any fluorescence donor or acceptor in order to study GPCR interactions via FRET. Furthermore, as a result of this invention, analysis of GPCR oligomerization and function *in vivo* via FRET has become the reliable, accurate method of choice for *in vivo* analysis of GPCRs. Thus, Applicant believes the claims are adequately enabled.

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending claims in this application is believed warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of this application to issue.

Payment in the amount of $210.00 (extension of time fee) is to be charged to a credit card and such payment is authorized by the signed, enclosed document entitled Credit Card Payment Form PTO-2038. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,



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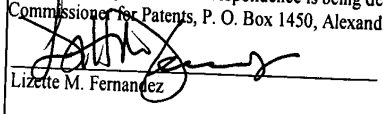

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